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14. ABSTRACT Uncertainty about the clinical behavior of a premalignant lesion can lead to either inappropriate inaction or inappropriate aggressive treatment, either of which can result in harm to the patient. The main goals of this DOD grant and program was to shed light on the molecular mechanisms of airway "field cancerization" and development of premalignant lesions. During this grant, we investigated and began to understand multiple mechanisms that lead to field of cancerization, including genomic aberrations and microRNA and gene expression changes. We have characterized the transcriptomic architecture of the adjacent airway field cancerization in early-stage NSCLC and validated the expression of a novel field cancerization markers in airways and tumors. Furthermore, we understood how the molecular field of injury evolves spatiotemporally and identified gradient profiles in the localized field cancerization that highly embody the nearby lung tumors. We identified field of injury/cancerization biomarkers that are specific to NSCLC vs benign disease, inform of NSCLC pathogenesis and can detect lung cancer when assessed in minimally invasive sites in the lung.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Project Summary.....	5
Aim 1.....	5
Aim 2.....	8
3. Key Research Accomplishments.....	9
4. Conclusion.....	9
5. Publications, Abstracts, and Presentations.....	9
6. Reportable Outcomes.....	9
8. References.....	10
7. Appendices.....	11

INTRODUCTION

Lung cancer continues to be the leading cause of cancer-related death in both men and women in the United States [1]. The majority of lung cancers are non-small cell lung cancers (NSCLCs) that include squamous cell carcinomas (SCCs) and adenocarcinomas [2]. Lung cancer mortality is high in part because most cancers are diagnosed after regional or distant spread of the disease had already occurred and due to the lack of reliable biomarkers for early detection and risk assessment [2]. The identification of new effective early biomarkers will improve clinical management of lung cancer and is linked to better understanding of the molecular events associated with the development and progression of the disease.

It has been suggested that histologically normal-appearing tissue adjacent to neoplastic lesions display molecular abnormalities some of which are in common with those in the tumors [3]. This phenomenon, termed field of cancerization, was later shown to be evident in various epithelial cell malignancies, including lung cancer [4, 5]. Loss of heterozygosity (LOH) events are frequent in cells obtained from bronchial brushings of normal and abnormal lungs from patients undergoing diagnostic bronchoscopy and were detected in cells from the ipsilateral and contralateral lungs [6]. More recently, global mRNA expression profiles have been described in the normal-appearing bronchial epithelium of healthy smokers [7]. In addition, modulation of global gene expression in the normal epithelium in health smokers is similar in the large and small airways and the smoking-induced alterations are mirrored in the epithelia of the mainstem bronchus, buccal and nasal cavities [8]. Finally, our group has previously shown that gene-expression profiles in cytologically normal mainstem bronchus epithelium can distinguish smokers with and without lung cancer and can serve as an early diagnostic biomarker for lung cancer⁹.

In this program, in Specific Aim 1, we extended our work in this field by spatially mapping the molecular field of injury associated with smoking-related lung cancer. In smokers undergoing resection of lung lesions, high-throughput mRNA expression analyses are being performed on cytological specimens (brushings) obtained at intraoperative bronchoscopy from the nasal epithelium, main carina and ipsilateral and contralateral proximal and distal bronchi (relative to the location of the resected lung lesion), as well as on specimens obtained at lobectomy from sub-segmental bronchus (adjacent to tumor) and from the resected NSCLC tumors. Towards this aim, we are comparing and contrasting global gene expression patterns across all the specimens from the entire field and corresponding NSCLC tumors. We have performed RNA-sequencing and microarray profiling of nasal epithelia, airway epithelial cells collected from both bronchoscopy and lobectomy specimens as well as of corresponding tumors (NSCLC patients) or benign lesions (cancer-free individuals).

In Specific Aim 2, we are using laser capture microdissection to obtain specific cell populations (basal cells or type II alveolar cells, depending on the NSCLC histology/location) as well as premalignant lesions and epithelial components of the tumors. These cell populations are being profiled with RNA-seq to determine their gene expression signatures to increase our understanding of premalignancy. We are analyzing the gene expression profiles that are associated with progression from a benign cell population to premalignancy and with progression from a benign cell population to true malignancy.

This report details the progress made during the fourth year of research.

Specific Aim 1: To increase our understanding of the molecular basis of the pathogenesis of lung cancer in the “field cancerization” that develops in current and former smokers.

Summary of Research Findings - AIM 1

Investigating molecular signature of lung cancer development in bronchial specimens by shotgun proteomics. A major challenge in reducing mortality from lung cancer hinges on identifying at-risk population. The bronchial epithelium may serve as surrogate tissue for biomarker of risk. Our objective was to identify a signature of risk from proteomic alterations in the cytologically normal airway epithelium from individuals at risk for developing lung cancer. We have identified 2792 protein, the largest to our knowledge, in bronchial brushings specimens collected from risk stratified individuals by shotgun proteomics. Joncheere-Terpstra trend test among low, medium and high risk groups resulted significantly altered expression of 316 proteins (trend $p < 0.05$) with 238 up and 78 down trends. Pathway enrichment analysis revealed up-regulation of carbohydrate metabolic pathways in the airways of high risk individuals. Expression of carbohydrate metabolic enzymes, lactate production and glucose consumption were increased in in vitro culture of human bronchial epithelial cells treated with cigarette smoke condensate. Up-regulation of these enzymes and other selected candidate proteins were validated by parallel reaction monitoring mass spectrometry and tissue microarray of histologically normal bronchial tissues from risk stratified individuals. We identified a signature of lung cancer risk assessment that may provide the basis of patient selection for surveillance programs and chemoprevention. Pathway enrichment analysis as well as in vitro data suggests possible metabolic reprogramming in the airway epithelium of high risk individuals.

Methods

Here we hypothesized that proteomic alterations in the histologically normal airway epithelium from risk stratified individuals allow us to derive a signature of risk of developing lung cancer.

Specimen collection procedure: Bronchial brushings were collected from patients under conscious sedation. The brushings were immediately dipped into 1.5 ml saline taken in a labeled eppendorf tube. The tube was kept on ice to minimize protease action. Care was taken to keep the brush specimen free

from blood. Brushings in the saline was vigorously agitated by vortexing for about 10 seconds with highest speed. It was then spun 1500g for 10 minutes in a microcentrifuge with the brush inside the tube. Supernatant was removed carefully leaving as little saline as possible keeping the brush inside the tube. The pellet was stored in freezer at -80°C temperature. Patients undergoing autofluorescence bronchoscopy for clinical

Table 1: Characteristics of individuals providing bronchial brushings for the training set

Characteristics		Risk levels		
		low (n=5)	Medium (n=5)	High (n=5)
Age	Average+stdev	58.2+4.7	59.4+10.7	72+5.2
	Median (range)	58 (53-65)	59 (47-74)	69 (68-78)
Gender	Male	4	2	3
	Female	1	3	2
Smoking status	Never smoker	5	0	0
	Ex-smoker	0	5	4
	Current smoker	0	0	1
	Pack year, avg+stdev	n/a	21.5+24.1	62.8+25.4
	Pack year, median (range)	n/a	20 (1-60)	50 (50-108)

suspicion of lung cancer agreed to provide bronchial biopsy specimens at predetermined normal sites (with normal fluorescence ratio). Biopsy specimens collected for research were snap frozen and stored in -80°C freezer. Patients characteristics are in Table 1.

Data acquisition: Proteins were extracted and fractionated as described previously. Equal amounts (micrograms) of proteins from each specimen of each group were pooled, divided into three technical replicates and each replicate was fractionated by isoelectric focusing (IEF). Shotgun proteomic data from each of the fifteen IEF fractions were collected by LC MS/MS (Velos). A combination of database search

tools were employed to identify peptide sequence present in a protein/protein groups. Normalized spectral counts of the digested proteins were considered as the basis of protein abundance measurement. Protein groups were identified by peptide matching, database search, filtration and parsimonious analyses using search tools developed at Vanderbilt University Medical Center (18-21). A protein group may include isoforms, variants or in rare occasions, unrelated proteins containing identical peptide sequence. Lisa and Rob to write the detail about sample preparation for LC MS/MS analysis. IEF fractionation of peptide digests and LC MS/MS data acquisition and analysis. Peptide identification from LC MS/MS data, protein assembly and filtering.

Statistical Methods: Joncheere-Terpstra Trend analysis Ming to write (groups 1, 2 and 3 Trend analysis). Validation of selected identified proteins by parallel reactions monitoring (PRM) analysis. Overexpressed carbohydrate metabolic enzymes in biopsies collected from individuals who provided bronchial brushings for the training set by Western blotting using matched biopsies.

Results

We have identified 2792 protein groups in the bronchial brushings specimen of risk stratified individuals by LC MS/MS shotgun proteomics (Table 2). Volunteers/subjects were categorized as low, medium and high risk groups using the lung cancer screening tool . Among the identified proteins in each group 50 to 60% were identified in each of the three replicates (Supplementary Table 4 and Supplementary Figure 2). Each protein was identified by two or more peptide sequences. Number of protein groups identified separately in groups 1, 2 and 3 were 2116, 1949 and 2771 respectively . Reproducibility of protein identifications in all three replicates were 49%, 48% and 61% in groups 1, 2 and 3 respectively.

spectral counts of each risk group			
Risk	Filtered	Distinct	Protein
Group	Spectra	Peptides	Groups
All groups			2792
Low	24637	5812	2116
Medium	24530	5115	1949
High	36598	9579	2771

To discover significantly dysregulated proteins in the bronchial epithelium of Individuals at risk for lung cancer development we performed Jonckheere-Terpstra trend test with 2526 identified proteins. Number of proteins with statistically significant (trend $p < 0.05$) increasing and decreasing trends were 316. Normalized spectral counts were used as quantitative measure of protein abundance. Expression of 238 out of 316 significantly dysregulated proteins demonstrated increasing trend and that of 78 proteins demonstrated decreasing trend.

Pathway enrichment analysis reveals profound alterations in metabolic enzymes regulating the glycolytic pathway and the TCA cycle. To understand the biological relevance of these alterations in the bronchial epithelium proteome pathway analysis was performed using Webgestalt (WEB-based GENE SeT Analysis Toolkit) (22). Because of the unavailability of larger human bronchial epithelium proteome we used our total identified proteins (2792 proteins) as the reference proteome of bronchial epithelium instead of entire human proteome. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis with 316 dysregulated proteins revealed dysregulation of metabolic pathways. Interestingly, enzymes of the glycolytic pathway, TCA cycle, pentose phosphate pathway, galactose, and glycogen metabolisms were overexpressed indicating very early events of possible metabolic reprogramming in the histologically normal bronchial epithelium of individuals at risk for lung cancer development (Figure 1). Moreover, six out of fourteen enzymes of the above mentioned pathways are rate limiting enzymes. Results of the Jonckheere-Terpstra trend test showing statistically significant increasing trend of the overexpressed carbohydrate metabolic enzymes among the risk groups using normalized spectral count of the replicates of each risk group are show in Figure 2.

Overexpression of ME2 may indicate diversion of excess TCA cycle intermediate malate to fatty acid biosynthetic pathway via pyruvate and acetyl coA. Among the 316 significantly altered proteins there were 49 metabolic enzymes. As expected enzymes of lipid metabolic pathways, fatty acid synthase (FASN), Acyl-CoA synthetase family member 3 (ACSF3) and arachidonate 15-lipoxygenase were also found to be overexpressed. Overexpression of alcohol and aldehyde metabolic enzymes ALDH1A1, ALDH3A1 and AKR1B10 in lung tumor tissues was reported enzymes to be overexpressed in the histologically normal bronchial epithelium of individuals at risk for lung cancer development.

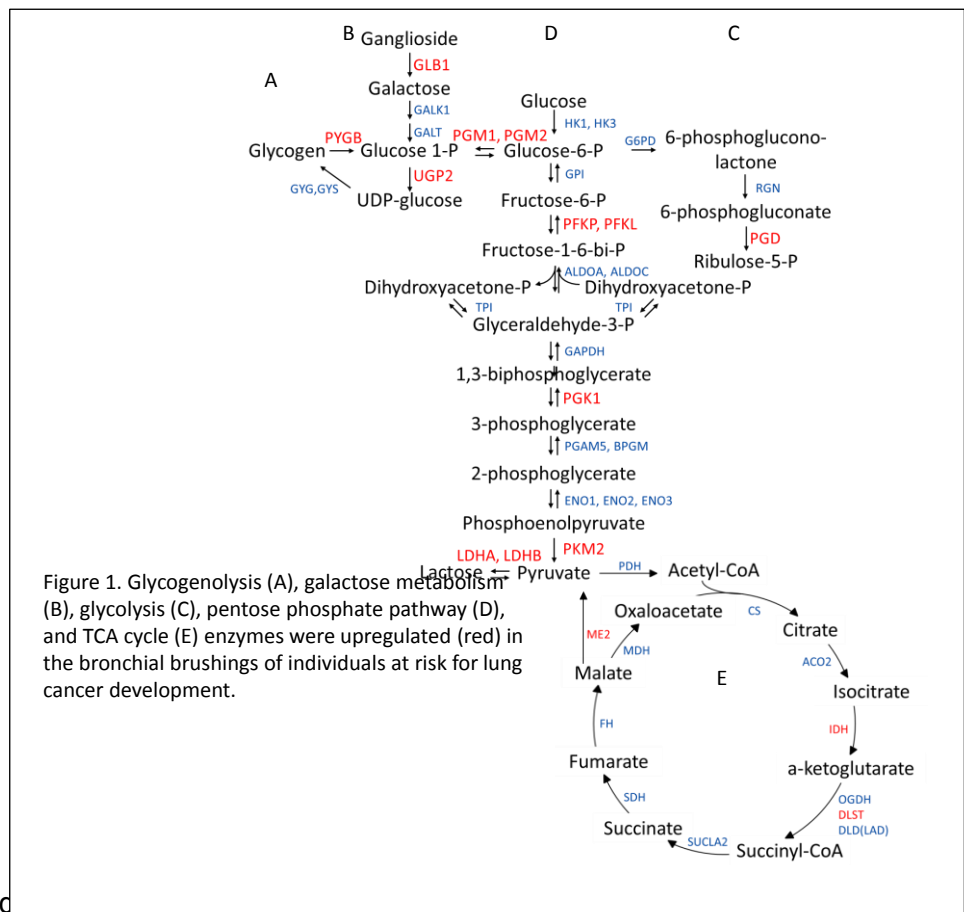


Figure 1. Glycogenolysis (A), galactose metabolism (B), glycolysis (C), pentose phosphate pathway (D), and TCA cycle (E) enzymes were upregulated (red) in the bronchial brushings of individuals at risk for lung cancer development.

Pathway analysis revealed metabolism as the top pathway to be dysregulated in individuals at risk for lung cancer development. The identification of ACSF3, ALOX15 and FASN in the bronchial epithelium of at risk individuals raises the possibility of involvement of lipid metabolism in early events of lung tumorigenesis. Previously we reported overexpression of acyl-coA binding protein/Diazepam binding inhibitor (ACBP/DBI) in lung cancer tissues indicating perturbation of lipid metabolism in lung cancer. To our knowledge, overexpression of the proteins identified and validated in the current study in the bronchial epithelium of individuals at high risk for developing lung cancer was not reported before.

Validation of over expression of carbohydrate metabolic enzymes and selected candidate proteins by PRM and immunohistochemistry in bronchial the epithelium. These experiments are in progress.

Warburg effect in the human bronchial epithelial cell lines by cigarette smoke condensate (CSC).

Given the overexpression of glycolytic enzymes including LDH we were curious to know if the level of lactate were altered in the airway epithelium of high risk individuals. As an alternative risk model we treated normal bronchial epithelial cell lines, 16HBE and BEAS2B with CSC in vitro and measured lactate and glucose concentrations in culture supernatant after 24 hours. Significantly enhanced lactate production and glucose consumption in vitro as a result of treatment of the cells with CSC (Figure 2) suggests a possible role of carbohydrate metabolic enzymes in lung cancer development. Whether these metabolic changes have any causative effect on the transformation of normal bronchial epithelial cells to dysplastic and finally malignant cells as Otto Warburg postulated more than half century ago remains to elucidated.

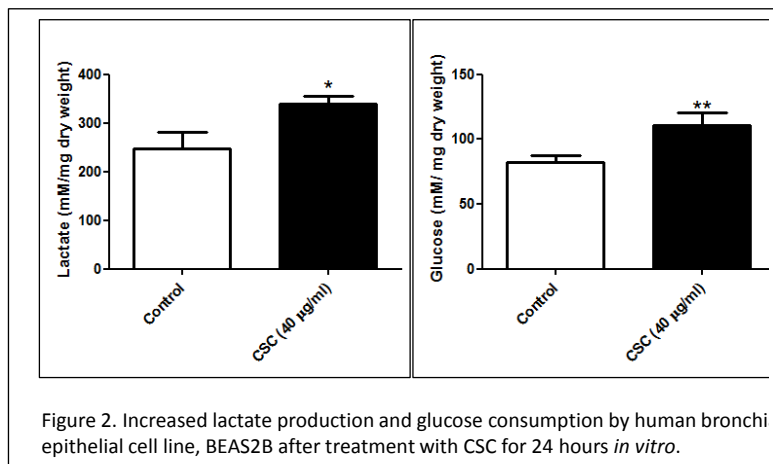


Figure 2. Increased lactate production and glucose consumption by human bronchi epithelial cell line, BEAS2B after treatment with CSC for 24 hours *in vitro*.

Specific Aim 2: Evaluate the role of airway epithelium tumor-initiating stem/progenitor cells in current and former smokers.

Summary of Research Findings – Aim 2

A. Assessment of the molecular profiles of tumor-initiating stem/progenitor cells from normal airway epithelium, premalignant lesions and cancer.

In collaboration with the Wistuba laboratory, the Massion laboratory has collected bronchial brushings from individuals at risk or with small cell lung cancer. We have collected on 13 individuals with SCLC, isolated total RNA and were able to obtain quality RNA on 11 of them as shown in Table 3. The RNA is part of a study to derive a signature for SCLC risk in the field of cancerization and to attempt to identify candidate tumor initiating progenitor stem cells in SCLC.

Table 3. Bronchial brushings from individuals diagnosed with SCLC yet untreated.

project 2967 #	Sample	Barcode	RIN	28s:18s	conc (ng/ul)	yield (ug)	SCLC
95	RNA	522214	7.9	1.3	260	3.9	X
86	RNA	519951	7.6	1.1	28	0.42	X
182	RNA	554960	7.6	1.3	283	4.245	X
177	RNA	515207	7.1	2.1	92	1.38	X
180	RNA	539943	7	1.9	52	0.78	X
68	RNA	524855	6.9	0.9	18	0.27	X
74	RNA	524354	6.7	0.9	247	3.705	X
181	RNA	532271	6.3	2	41	0.615	X
179	RNA	555886	2.4	2.1	68	1.02	X
178	RNA	522995	2.3	1.7	130	1.95	X
183	RNA	526992	2.3	1.5	110	1.65	X

KEY RESEARCH ACCOMPLISHMENTS

During our fourth year of research, we followed up on our successful mRNA and Proteomic sequencing from bronchial airway epithelial cells from individuals at various risk for lung cancer. Based on linear trend analysis we discovered that metabolic reprogramming is happening in non transformed cells from individuals at high-risk for lung cancer. Specifically we identified evidence for the Warburg effect with increase in glucose uptake and Lactate production. This reprogramming was associated with overexpression of rate limiting enzymes including PFKF, PKM2, IDH1, IDH2 and PYGB. We verified this observation by Parallel Reaction Monitoring in an independent set of 30 individuals at various risk for lung cancer and in vitro in a model of cultured airway epithelial cells exposed to cigarette smoke condensate (40 microgram/mL). We concluded that the trend analysis of proteomic survey of bronchial epithelium at increasing risk for lung cancer reveals major metabolic enzyme reprogramming. CSC induces metabolic reprogramming in HBEs and that the Warburg effect is observed before transformation with rate limiting metabolic enzymes being dysregulated. Most of the metabolic enzymatic alterations are targets of Nrf2 regulation and are likely contribute to tumorigenesis.

CONCLUSIONS

- Bronchoscopy specimens like brushings can be used successfully for shotgun proteomic profiling and used to derive a signature of risk for lung cancer development.
- Differential protein expression of proteins following an increased risk for lung cancer was demonstrated in bronchial brushings by shotgun proteomics.
- We identified a signature of lung cancer risk assessment that may provide the basis of patient selection for surveillance programs and chemoprevention. Pathway enrichment analysis as well as in vitro data suggests possible metabolic reprogramming in the airway epithelium of high risk individuals. This work has important implications as potential biomarkers of risk and in the potential design of new chemopreventive strategies in high risk individuals.

REPORTABLE OUTCOMES

Abstracts:

Rahman SMJ, Li M, Zou Y, Zimmerman ML, Lu P, Slebos R, Zhang B, Shyr Y, Liebler D and Massion PP. Proteomic analysis of the bronchial epithelium to develop a molecular signature of risk for lung cancer. IASLC 15th World Conference on Lung Cancer, Sydney, Australia, October, 2013. (ID 2008)

Jun Qian, Xiangming Ji, Yong Zou, Megan D. Hoeksema, Heidi Chen and Pierre P. Massion
Overexpression of miRNA-1224-5p, 1248 and 944 on chromosome 3q26-29 in squamous cell carcinoma of the lung. 1. 2014 AACR meeting abstract (#4989):

Manuscripts submitted or in preparation:

Jun Qian, Mohamed Hassanein, Megan D. Hoeksema, Bradford K. Harris, Yong Zou, Heidi Chen, Pengcheng Lu, Rosana Eisenberg, Jing Wang, Allan V Espinosa, Xiangming Ji, Frederick T. Harris, S.M. Jamshedur Rahman and Pierre P. Massion. The RNA binding protein FXR1 is a new driver in the 3q26-29 amplicon and predicts poor prognosis in human cancers (Under revisions)

Rahman SMJ, Li M, Zou Y, Zimmerman ML, Lu P, Slebos R, Zhang B, Shyr Y, Liebler D and Massion PP. Proteomic analysis of the bronchial epithelium to develop a molecular signature of risk for lung cancer. (Manuscript in preparation)

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Publications and Meeting Abstracts

YEAR1

Manuscript:

1. **Gomperts BN**, Spira A, Massion PP, Walser TC, Wistuba II, Minna, JD and Dubinett SM. Evolving concepts in lung carcinogenesis. *Seminars in Respiratory and Critical Care Medicine*. Semin Respir Crit Care Med. 2011 Feb;32(1):32-43. Epub 2011 Apr 15. PMID: 21500122

Abstracts:

1. Kadara H, Saintigny P, Fan Y, Chow CW, Chu ZM, Lang W, Behrens C, Gold K, Liu D, Lee JJ, Mao L, Kim ES, Hong WK, Wistuba II. Gene expression analysis of field of cancerization in early stage NSCLC patients towards development of biomarkers for personalized prevention. Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract #3674.
2. Wistuba I, Kadara H, Kim ES, Hong WK. Molecular Pathology of Lung Cancer & Intermediate Markers of Carcinogenesis. 14th World Conference on Lung Cancer; 2011. Abstract #M19.

YEAR 2

Abstracts:

1. Ooi AT, Gower AC, Zhang KX, Vick J, Caballero N, Massion PP, Wistuba II, Walser TC, Dubinett SM, Pellegrini M, Lenburg ME, Spira A and Gomperts BN. Molecular Profiles to Improve our Understanding of Lung Cancer Pathogenesis in U.S. Veterans. NIH Lung Cancer SPORE Meeting. Pittsburgh. July 2012

YEAR 3

Abstracts:

1. Maki Y, Fujimoto J, Yoo SY, Gower A, Shen L, Garcia MM, Kabbout M, Chow CW, Hong WK, Kalhor N, Wang J, Moran C, Spira A, Coombes KR, Wistuba II, Kadara H. Transcriptomic architecture of the airway field cancerization in early-stage non-small cell lung cancer. 104th Annual American Association for Cancer Research (AACR) meeting, April 6 - April 10 2013, Washington, D.C. Abstract # 2367.
2. Ooi AT, Gower AC, Zhang K, Vick J, Hong LS, Fishbein M, Nagao B, Wallace WD, Elashoff DA, Dubinett S, Lenburg M, Spira A, Gomperts BN. Gene expression alterations in premalignant lesions from the airways of patients with lung squamous cell carcinomas. Platform presentation and travel award. AACR Washington DC, April 2013.

Manuscripts:

1. Ooi AT, Gower AC, Zhang KX, Vick JL, Hong L, Nagao B, Wallace WD, Elashoff DA, Walser TC, Dubinett SM, Pellegrini M, Lenburg ME, Spira A, Gomperts BN. Profiling premalignant lesions in lung squamous cell carcinomas identifies mechanisms involved in stepwise carcinogenesis. Cancer Research, submitted and under revision.
2. Kadara H, Fujimoto J, Yoo SY, Maki Y, Gower AC, Kabbout M, Garcia MM, Chow CW, Chu Z, Mendoza G, Shen L, Kalhor N, Hong WK, Moran C, Wang J, Spira A, Coombes KR, Wistuba II. Transcriptomic architecture of the adjacent airway field cancerization in non-small cell lung cancer. Journal of the National Cancer Institute. Submitted and Under Revision.
3. Perdomo C, Campbell JD, Gerrein J, Tellez C, Garrison CB,, Walser TC, Drizik E, Si H, Gower AC, Vick J, Anderlind C, Jackson JR, Mankus C, Schembri F, O'Hara C, Gomperts BN, Dubinett SM, Hayden P, Belinsky SA, Lenburg ME, Spira A. miR-4423 is a Primate-Specific Regulator of Airway Epithelial Cell Differentiation and Lung Carcinogenesis. Proc Nat Acad Sci USA, accepted.

4.

YEAR 4

Abstracts:

1. Rahman SMJ, Li M, Zou Y, Zimmerman ML, Lu P, Slebos R, Zhang B, Shyr Y, Liebler D and Massion PP. Proteomic analysis of the bronchial epithelium to develop a molecular signature of risk for lung cancer. IASLC 15th World Conference on Lung Cancer, Sydney, Australia, October, 2013. (ID 2008)
2. Jun Qian, Xiangming Ji, Yong Zou, Megan D. Hoeksema, Heidi Chen and Pierre P. Massion Overexpression of miRNA-1224-5p, 1248 and 944 on chromosome 3q26-29 in squamous cell carcinoma of the lung. 1. 2014 AACR meeting abstract (#4989):

Manuscripts submitted or in preparation:

1. Jun Qian, Mohamed Hassanein, Megan D. Hoeksema , Bradford K. Harris , Yong Zou, Heidi Chen, Pengcheng Lu, Rosana Eisenberg, Jing Wang, Allan V Espinosa, Xiangming Ji, Frederick T. Harris, S.M. Jamshedur Rahman and Pierre P. Massion. The RNA binding protein FXR1 is a new driver in the 3q26-29 amplicon and predicts poor prognosis in human cancers (Under review)
2. Rahman SMJ, Li M, Zou Y, Zimmerman ML, Lu P, Slebos R, Zhang B, Shyr Y, Liebler D and Massion PP. Proteomic analysis of the bronchial epithelium to develop a molecular signature of risk for lung cancer. Manuscript in preparation

Project Personnel Report	
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Molecular Profiles for Lung Cancer Pathogenesis and Detection in U.S. Veterans	
All personnel who received pay from the project - Yr 4 personnel in bold.	
Massion, Pierre	
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